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THE PROTEOLYTIC ACTIVITIES OF THE PANCREATIC JUICE.

By W. M. BAYLISS and E. H. STARLING.

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It is remarkable that although one of the earliest of the modern researches on the pancreatic juice, namely, that of Corvisart[1], was made on the pancreatic juice itself as secreted into the duodenum, nearly the whole of our modern ideas on this subject are based on experiments carried out, not on the juice, but on extracts made in various ways from the gland itself. It is true that the observations of Claude Bernard[2] had reference to the actions of the juice collected from a fistula, but the omission of any reference in his results to a proteolytic activity of this juice has tended to depreciate the importance of his work in the light of the later experiments of Kühne, Heidenhain, Langley, and others on extracts of the gland. Corvisart noted chiefly the action of the juice on proteids, Claude Bernard mentioned its amylolytic action, but devoted chief attention to its power of splitting up and saponifying fats. The work of Kühne reestablished in the minds of physiologists the importance of this gland as a proteolytic agent. Heidenhain and Langley showed that the proteolytic ferment trypsin was in the fresh gland present, not preformed, but in the form of a precursor, trypsinogen. Pancreatic juice itself, though employed by Heidenhain in a few experiments, has been the subject of detailed investigation only since Pawlow elaborated his method of establishing permanent pancreatic fistulæ in dogs. It will be remembered that in this method the patch of duodenum on which the pancreatic duct opens is brought to the surface and stitched into the abdominal wound. In this way, whenever the dog is fed a flow of pancreatic juice is obtained which, trickling from the duct over the small patch of mucous membrane, drops down and can be received into a funnel, and so into a receiver tied to the belly of the animal. In showing that this juice possessed the three

[1] *Sur une fonction peu connue du pancréas*, Paris. 1857.

[2] *Liquides de l'organisme*, T. II. Leçons, 14 and 15. Paris. 1858.

ferment actions which have been attributed to it since the time of Kühne, pupils of Pawlow, especially Walther[1], drew attention to the marked differences in the juice caused by variations in the diet of the animal, and asserted that according as the diet contained chiefly proteid, fat, and carbohydrate, so did the contents of the juice in trypsin, steapsin, or amylopsin respectively increase. The results of these observers, especially in the case of trypsin, showed, however, some discrepancies. The reason for this was apparent when Chepowalnikow[2] showed that the pancreatic juice obtained in Pawlow's mode of procedure contained only a small portion of trypsin, but a large amount of trypsinogen, and that this latter substance needed for its conversion into trypsin the interaction of a body contained in the succus entericus, and called by Pawlow enterokinase. According to Pawlow enterokinase acts as a 'ferment of ferments,' *i.e.*, acting in minimal quantities, converts the trypsinogen into trypsin, just as trypsin converts proteid into its hydrated products.

Quite lately, Delezenne and Frouin[3], working in the Pasteur Institute, have found that the pancreatic juice, if drawn from a cannula in the duct so as to avoid any contact with the little patch of mucous membrane forming the floor of the fistula, is perfectly free from trypsin, and that this body is only formed from the trypsinogen in Pawlow's experiments as the juice trickles from the mouth of the duct over the mucous membrane. This author has also put forward an explanation of the action of enterokinase which differs from the hypothesis of Pawlow.

We shall later on have to consider these hypotheses, as also the statement of Delezenne[4] concerning the origin and distribution of enterokinase itself. Our experiments were directed to obtain an exact knowledge of the properties of the juice itself as a preliminary to an investigation of the adaptive powers of the gland which had been described by Walther. They have extended over a longer time than we expected, owing to the complexity of the points involved and the defective state of our knowledge concerning the intimate nature of ferment action. They have, however, been rendered easier by the discovery of secretin, and the easy method thereby afforded of obtaining pancreatic juice at any time, and from an animal in any condition, without the formalities of making a permanent fistula.

[1] See Pawlow. *Le travail des glandes digestives.* Paris. 1901.
[2] Thèse (Russian). [illegible]etersburg. 1899. Paris. 1901.
[3] *C. R. Soc. de Biol.* 1902, p. 691. Paris. [4] *Ibid.*, p. 281. Paris.

Experimental Methods.

In nearly all cases dogs were used as the source of juice. In all cases the animals were anæsthetised with morphia and A.C.E. A cannula being tied in the pancreatic duct, juice was obtained by injecting at intervals of 10 minutes solutions of secretin obtained in the manner described in a former paper[1]. As a rule the intestines of each dog were used for the preparation of secretin to be injected into another dog on a subsequent day. The cannula and rubber tube attached were always boiled before the experiment, the juice was received into sterile flasks, the first few drops of secretin, representing the juice which had been stagnant in the ducts, being rejected. The collection of juice was kept up from 4 to 12 hours, varying quantities of juice, from 30 to 120 c.c. being obtained from each dog. It was found that the animal kept in better condition if the secretin solutions (which contained in nearly all cases also the depressor substance) were injected in small doses, 1 to 2 c.c., frequently, than when larger doses were injected at longer intervals.

In a few cases a secretion of juice was obtained by the injection of pilocarpine. This drug differs markedly from secretin in its action on the pancreas. Injected in a dose from 10 to 15 mgrms. into a large dog it caused a flow of juice much slower than that obtained by secretin. This flow might not begin till 5 or 10 minutes after the injection, and lasted a-half to one hour. Sometimes a second injection invoked a second but smaller flow of juice, with the same time relations. But in many cases the second dose was ineffective. In all cases the gland seemed to rapidly tire and become insusceptible to the drug. The juice obtained by pilocarpine is much thicker and richer in solids than that given by secretin. It is possible that part of the difference between the two methods of gland stimulation is conditioned by the local vaso-constriction, which is one of the effects of pilocarpine injection. There are several other points of difference, however, between the action of these two chemical agents, as mentioned in our previous paper. In many cases, where it was required to keep the juice for some time, it was at once mixed with an equal volume of 2% sodium fluoride, and the mixture was kept in the ice-safe until required for observation. The fresh juice, however, does not possess any marked tendency to

[1] This *Journal*, XXVIII. p. 333. 1902.

putrefy, and if collected in a clean vessel may often be kept for days without any sign of decomposition.

In order to test the proteolytic activity of the juice under various conditions, we made use of the methods devised by Mett and by Fermi.

Mett's method implies the digestion of coagulated egg-white in small glass tubes at a temperature of 35° C. to 40° C. We found that this treating of the juice, however, materially altered its activity. The process of auto-destruction of the tryptic ferment is so rapid at this temperature, especially if the fluid be fairly free from dissolved proteids, that the ferment may undergo destruction before it has had time to digest any large quantity of the egg-white.

On this account the larger number of our observations were made by Fermi's method, using tubes filled with coloured gelatin (made with 1 % NaF solution), and allowing them to digest at the temperature of the room. Since all our observations were comparative, each group being carried out at one time, alterations in the temperature of the room did not vitiate our results, since all the specimens under observation were affected simultaneously. Under these conditions a juice may be more active at the end of a week than it would be after 5 hours at a temperature of 40° C.

In all our experiments the number of millimetres given imply the average amount digested at each end of 2 or 3 tubes during the time stated.

The mode of preparation of the solutions of enterokinase used by us is given in a later section of this paper.

General characters of the pancreatic juice.

Juice obtained by the injection of secretin is a clear or very slightly opalescent fluid, only slightly viscid, and very strongly alkaline. It contains from 1·5 to 2·5 % of solids, of which about 1 % is represented by salts, chiefly sodium carbonate, and the other ·5 to 1·5 % by proteids. Its alkalinity is equal to or greater than a decinormal sodium carbonate solution. Thus in some experiments 10 c.c. of the juice needed for its neutralisation 15 c.c. of $\frac{N}{10}H_2SO_4$, and in no case did we find its alkalinity less than decinormal. The proteids of the fresh juice all belong to the class of coagulable proteids, and are totally precipitated by trichloracetic acid. On neutralising the juice a precipitate is produced

which is largely increased on heating to 60° C.; fully 50 % of the proteids are precipitated at this temperature.

Pilocarpine juice contains from 3 % to 5 % total solids, the chief difference between it and the secretin juice being determined by proteids. Its alkalinity is generally somewhat less than that of secretin juice. Qualitatively, the proteids seem to resemble those of secretin juice. A detailed chemical examination of both kinds of juice is, however, being made by Mr de Zilwa. The above short account will therefore serve the purposes of the present paper.

The digestive properties of secretin juice.

Fresh juice obtained as above has a strong amyolytic action on starch, and a slight fat-splitting action, which is largely increased by the presence of bile. On certain proteids it has a feeble but definite action. Fresh fibrin, if allowed to remain in contact with the juice at a temperature of 38° C., is apparently unaltered at the end of one or two hours; but is completely dissolved at the end of 24 hours. This action on fibrin has been ascribed by Delezenne[1] to the presence in the fibrin of enterokinase derived from disintegration of leucocytes. We shall show later on that enterokinase is completely destroyed at 65° C. Fibrin, however, can be heated to 70° C. for a quarter or half-an-hour, without losing altogether its solubility in pancreatic juice. The fibrin at this temperature is coagulated to a certain extent. When treated with pancreatic juice it will still dissolve, but now will require 3 to 4 days instead of the one day required by fresh fibrin. Pancreatic juice also hydrolyses caseinogen, though slowly, and apparently the proteolytic ferment in the juice attacks the proteids of the juice itself. Thus if the juice has been kept for some time, and then treated with trichloracetic acid, the filtrate will give a slight but distinct biuret reaction. Pancreatic juice is without effect on coagulated proteid, or on solid gelatin at the temperature of the room. Coagulated egg albumen may be kept in contact with the juice for a week without undergoing solution. Fresh pancreatic juice, in fact, resembles closely in its proteolytic functions the succus entericus. It has been shown by Kutscher and Seemann[2] that the erepsin described by Cohnheim[3]

[1] *C. R. Soc. de Biol.* 1902, p. 591.
[2] *Zeitsch. f. physiol. Chemie.* xxxv. p. 445. 1902.
[3] *Op. cit.* xxxiii. p. 464. 1901.

does not confine its action to albumoses and peptones but has also a feeble solvent action on fibrin and on caseinogen. Fresh juice may be kept for weeks without any apparent alteration in this feeble proteolytic power. Hence it is evident that its power is not due to a partial spontaneous conversion of the trypsinogen into trypsin. This latter potent ferment apparently makes no qualitative difference between coagulated and dissolved or uncoagulated proteid. It may be so largely diluted that its action on fibrin is reduced to the feeble degree which characterises fresh pancreatic juice. In this dilution it still digests coagulated egg-white and gelatin slowly, whereas fresh pancreatic juice which will digest fibrin in 24 hours is without effect on coagulated egg albumen or globulin, even after the lapse of a week. This is shown by the following experiment.

Feb. 2nd. (*a*) 10 c.c. 1 % NaF + 0·1 c.c. solution of Grübler's trypsin and gelatin tubes at 15° C.

(*b*) 10 c.c. 1 % NaF + 0·1 c.c. solution of Grübler's trypsin and fibrin at 38° C. After 24 hrs. 1·1 mm. of the gelatin was digested, and the fibrin was about half gone. After a further period of 24 hrs. the gelatin tubes were digested to the extent of 3 mm., while the fibrin was completely dissolved.

We believe therefore that the outgrowth of the intestinal epithelium which gives origin to the pancreas retains one primitive function of the intestinal epithelium, namely, the power of secreting a very feeble proteolytic ferment, and should describe the pancreatic juice as containing a ferment similar to that which was described by Cohnheim in the succus entericus and named by him erepsin. It is possible that the feeble proteolytic ferments described by various observers in Brunner's glands and as pseudo-pepsin are of the same nature.

Very different is the case when a trace of succus entericus or extract of intestinal mucous membrane is added to the fresh juice. Within a short time the juice becomes the most powerful proteolytic agent with which we are acquainted, one which, at body temperature, will digest fibrin within 10 minutes, and tubes of coagulated egg-white within a few hours. The juice acquires on this addition those proteolytic qualities which have been ascribed to it since the time of Kühne, and which we associate with the presence of trypsin. The succus entericus or the intestinal mucous membrane may be so diluted as to possess no proteolytic powers, and yet in maximal dilution is able to activate large quantities of the juice.

Thus in an experiment (Dec. 15, 1902), 5 c.c. of fresh juice was

treated with 0·008 c.c. of a solution of enterokinase, which had already been found to be devoid of any proteolytic power when tested on gelatin, and the gelatin tubes added. After 20 hours at room temperature 5·1 mm. were digested. Fresh tubes were then added, and after another 24 hours these were digested to the extent of 7·2 mm. Control experiments were made with the juice without enterokinase—but the tubes showed no trace of solution, even after three days.

Nature of action of enterokinase.

As we mentioned above, Pawlow regarded enterokinase in the light of a ferment. Delezenne[1], on the other hand, has sought to bring the interaction of these digesting juices into line with the adaptation studied by bacteriologists in the formation of antitoxins, hæmolysins, etc. According to Delezenne, enterokinase belongs to the class of cytases which have been described by Metchnikoff, and identified by him as products of the various types of phagocytes, especially the white blood corpuscles. These bodies are of almost universal occurrence, and are identical with those described by Buchner as alexins, and by Ehrlich as complements. These cell-dissolving substances cannot act on their object of attack without an intermediary substance which shall anchor them on to the substance in question. This intermediary substance, the immune body, or anti-body, is specific, and varies with the substance to be attacked, *e.g.* red blood corpuscles. Metchnikoff and Delezenne regard the trypsinogen of the pancreatic juice as this intermediary body or amboceptor, which can lay hold of the proteid molecule on the one hand, and of the cytase or enterokinase on the other, and so permit the latter to attack and break up the proteid molecule. It is evident that these two views are diametrically opposed to those of Pawlow. Delezenne has sought to support his view by the observation that the enterokinase can be abstracted from a fluid by means of fresh fibrin, just as the immune bodies can be abstracted from blood by red corpuscles in the cold, and be precipitated with them. The two views are, however, so fundamentally distinct that no difficulty is experienced in devising experiments which shall decide between the two. In the first place, according to Delezenne's view there must be always a quantitative proportion between the amount of enterokinase and the

[1] *C. R. Soc. de Biol.* 1901, p. 1161.

amount of trypsinogen, each "molecule" of enterokinase will need a "molecule" of trypsinogen to anchor it on to the proteid molecule. The amount of enterokinase which may be added to a given solution of trypsinogen to impart the optimum proteolytic power to the solution will be reached directly the number of molecules of enterokinase equals that of the trypsinogen. This condition, once attained, will be permanent, and under no condition could we expect by the use of a minimum quantity of enterokinase to evoke a maximum proteolytic activity by allowing a longer time for the interaction to take place. On the other hand, this is precisely what would happen if the enterokinase acted as a ferment. A larger amount of enterokinase would convert the trypsinogen rapidly into trypsin, while the same effect should be produced by a much smaller amount of enterokinase if only interaction were allowed to go on for a much longer time.

We therefore, in the first place, instituted a number of experiments on the quantitative relationships of enterokinase to trypsinogen. Fresh pancreatic juice, with or without the addition of sodium fluoride, was treated with quantities of enterokinase varying from ·0001 c.c. up to an equal bulk, and the action of the mixture at once tested on gelatin tubes and on coagulated egg-white. It was found that if the experiment was allowed to go on for two days before interruption, that the differences between the results were only slight, and that it was very difficult to determine any optimum degree for the amount of enterokinase. On the other hand, if the observations of the tubes were made repeatedly at short intervals it was found that at first there was a very marked difference in the activities of the various mixtures, those containing a large amount of enterokinase being very active, while those containing the smallest quantities might be absolutely inactive. Later on these differences gradually disappeared. There was a rapid increase in the proteolytic activity of the solutions containing the traces of enterokinase, while, at room temperature, those containing larger amounts slowly diminished in activity. These results are well illustrated in the following experiment, which may serve as a type of the large number we made on this point.

Oct. 21st. Mixtures made in sterilised flasks of various proportions of fresh pancreatic juice (J), a sodium fluoride extract of intestinal mucous membrane (Ent) and 1 % sodium fluoride solution. These were treated with gelatin tubes and the digestion observed at the end of 6 hrs. and 24 hrs. Fresh tubes were then added, and a 3rd set of readings taken after another 24 hrs. The results are shown in the following table.

	Flasks.		Length of Gelatin digested in mm. After 6 hrs.	24 hrs.	A further 24 hrs. (fresh tubes).
A.	(4)	·5 c.c. J. + 4·4 1% NaF + 0·1 Ent.	1	5	5
	(5)	„ „ 4·1 „ „ 0·4 „	2	6·5	5·25
	(6)	„ „ 2·9 „ „ 1·6 „	3	7·5	5
B.	(7)	2 c.c. J. + 3 NaF + 0·1 Ent.	0	7	7·25
	(8)	„ „ 2·6 + 0·4 „	2	9	7
	(9)	„ „ 1·4 1·6 „	3	12	7
C.	(10)	3·4 c.c. J. + 1·6 NaF + 0·1 Ent.	0	9	8·5
	(11)	„ „ 1·2 „ 0·4 „	2·25	12	8·25
	(12)	„ „ 0 „ 1·6 „	3·2	13	8·75
D.	(1)	5 c.c. J. + 1 NaF + 0·03 Ent.	0	·5	7·75
	(2)	„ „ 0·9 „ 0·1 „	0	7·5	10
	(3)	„ „ 0 „ 1·0 „	3	12	8·5
E.	(13)	10 c.c. J. + 1·5 NaF + 0·1 Ent.	0	4·1	10·5
	(14)	„ „ 1·1 „ 0·4 „	2	12	9·25
	(15)	„ „ 0 „ 1·6 „	3	14	9

Gelatin tubes added to all.

So far these experiments confirm Pawlow's view, the enterokinase resembling in its action a ferment, *i.e.*, acting directly on the trypsinogen, and not in coöperation with an equal number of trypsinogen molecules on the proteid molecule.

If Delezenne's view were correct, every known trypsin must owe its activity to the coexistence in it of the two bodies trypsinogen and enterokinase. It becomes therefore of importance to seek for some means of determining the presence or absence of these two substances in the commercial and other preparations of trypsin. We hoped at first that we should be able to decide this question by studying the effect of heat on trypsin solutions. If we take three solutions, namely, fresh juice, enterokinase, and a solution of Grübler's trypsin, we find that whereas the trypsin and enterokinase are entirely destroyed at 65° C., the pancreatic juice does not lose its power of forming an active solution with enterokinase until it is heated to 70° C., that is to say, trypsinogen will stand a higher temperature than either trypsin or enterokinase. This difference, however, is not sufficiently absolute to enable us to decide the question as to the presence or absence of enterokinase and trypsinogen in the commercial extracts, since trypsinogen is considerably damaged by exposure to a temperature of 65° or 66°, while in certain conditions of the solution, as, *e.g.*, Benger's liquor pancreaticus, the trypsin may be heated as high as 70° C. without losing all its power. These results are shown by the following experiments.

Oct. 6th, 1902. Relative resistance to heat of Trypsinogen, Enterokinase, and "Trypsin" (*i.e.* juice and enterokinase which had been kept for 1 hr. at 35° C.).

Controls.

	Digestion after 17 hrs.
Juice alone with gelatin tubes	0
Activated juice with gelatin tubes	9
Enterokinase alone with gelatin tubes	0

Trypsinogen.

	Digestion after 17 hrs.
5 c.c. juice alone heated to 60° C. for 10 mins., cooled and enterokinase added, with gelatin tubes	7
" " " 65° " " " "	7
" " " 70° " " " "	0

Enterokinase.

	Digestion after 17 hrs.
5 c.c. juice + enterokinase previously heated for 5 mins. to 55° C.	8·5 mm.
" " " " " 60° C.	3 "
" " " " " 65° C.	0 "

"Trypsin."

5 c.c. activated juice heated to 60° C. for 10 mins.	4 mm.
" " " 65° C. "	2 "
" " " 70° C. "	0 "

The inactive juice became cloudy at 60° C. The enterokinase solution gave a coagulum at 55° C. The activated juice did not become cloudy on heating.

In another experiment on various commercial preparations of trypsin all became inactive at 65° C. except Benger's, which retained a slight activity even after heating to 75° C.

One criterion which, in conjunction with other results, is of importance, is that afforded by the auto-destructibility of these substances. Fresh juice can be kept at 40° C. for many days, and will still, when treated with enterokinase, give an active juice. Trypsin solution, solutions of enterokinase, or mixtures of the two, are very rapidly destroyed at this temperature, although fairly stable at the ordinary room temperature of 16° C.

This is shown by the following experiments:—

Effect of keeping at 16° C. and at 38° C. on trypsinogen and activated juice.

Tube		Digestion after successive periods of 24 hrs.		
(1)	5 c.c. juice + 1 c.c. enterokinase + gelatin tube at 16° C.	7·8	5	5·5 mm.
(2)	" " at 38° C. for 24 hrs.		+ tube 2	2 "
(3)	" " " for 48 hrs.			+ tube ·5 "
(4)	" " at 15° C. for 48 hrs.			+ tube 5·6 "
(5)	5 c.c. juice alone at 38° C. for 24 hrs.	+ enterokinase and tube 8·25		7·25 "
(6)	" " " for 48 hrs.	+ enterokinase + tube 8		"
(7)	" " at 16° C. for 48 hrs.	+ enterokinase and tube		7 "

In this experiment the testing of the digestive powers of the juice on gelatin were of course carried out at the room temperature (about 16° C.), whereas in No. (2) 24 hrs. at 38° C. had diminished the amount digested in 24 hrs. to ¼, and 48 hrs. had almost completely abolished the proteolytic power of the mixture of juice and enterokinase; in No. (6) the heating of the juice alone to this temperature for 48 hrs. had absolutely no effect on its activity when subsequently treated with enterokinase.

Effect of keeping at 38° C. on enterokinase.

Oct. 21st. (1) Enterokinase in incubator.
(2) ,, room temperature.
(3) 5 c.c. juice + 1 c.c. enterokinase at 38° C.

After 24 hrs. flasks were prepared containing

(*a*)	5 c.c. fresh juice + 1 c.c. enterokinase (1)	after 24 hrs. digestion	1	mm.
(*b*)	,, ,, ,, ,, (2)		10·25	,,
(*c*)	The same as No. (3)		1·5	,,

All had gelatin tubes added.

The rapid diminution in power of active solutions of trypsin has already been shown by Vernon. We have fully confirmed this observer's results, using different commercial preparations of trypsin, as well as active glycerine extracts of the gland.

It is worthy of note that the auto-destruction of trypsin is most marked in pure solutions of this body, and is markedly diminished by the presence of proteids or hydrated proteids in the solutions. It is as if the trypsin, deprived of molecules to attack, turned its destructive powers on to itself.

This power of preventing the auto-destruction of the trypsin is noticeable even with small quantities of proteid such as is contained in the juice itself; on this account Grübler's trypsin in watery alkaline or NaF solutions loses its power more rapidly than activated pancreatic juice.

The following experiments show the protection afforded by the presence of a little egg albumen (egg-white diluted 10 times with distilled water filtered and boiled) on Grübler's trypsin, and on activated juice.

I. Grübler's trypsin (solution in 1 % NaF).

(*a*) 3 c.c. trypsin + 10 c.c. boiled egg albumen + 10 c.c. 2 % NaF at 40° C. for 24 hrs.
(*b*) ,, + 10 c.c. water + ,, ,, ,,
(*c*) ,, ,, + ,, at 15° C. ,,

Then added to each gelatin tubes, and kept at room temperature

	Digestive power	
	After 24 hrs.	After 48 hrs.
(*a*)	1·5 mm.	4·5 mm.
(*b*)	0 ,,	1·5 ,,
(*c*)	3 ,,	7 ,,

II. Activated juice.

(α)	5 c.c. juice + 1 c.c. enterokinase + 5 c.c. boiled egg-white	at 40° C. for 3 days	
(β)	,, ,, + 5 c.c. water	,,	,,
(γ)	,, ,, ,,	at 15° C.	,,

Gelatin tubes then added.

Digestion after 24 hrs.
8 mm.
1 ,,
13 ,,

The protective influence of proteids seems to vary within wide limits with the amount of proteid present. Thus it is still more marked when the trypsin solutions are treated with an equal bulk of 5 % casinogen solution, or a 10 % solution of commercial peptone, than in the two experiments just quoted. It is remarkable that "peptone" solutions should have this preservative effect. It is generally stated and believed that the action of trypsin as of other ferments is inhibited by the accumulation of the products of its action. In this case a solution of trypsin containing peptones, etc. closely allied to the products of its activity, when tested on tubes of coagulated egg-white at 40° C. will apparently excite a much stronger digestive action than another similar solution without such admixture. In comparing the activities of any two solutions we have therefore to take into account not only the amount of ferment present, and the reaction of the solution, but also the amount of proteids or the products of their digestion present in the two fluids.

It is evident that according to Delezenne the activity of a tryptic solution is due to the coexistence in it of trypsinogen and enterokinase.

If therefore we can by any means exclude the presence of enterokinase from such a solution we can be equally certain that its activity must be due not to trypsinogen but to a third substance, namely, *trypsin*.

The distinguishing character of enterokinase, in fact the only character by which we can judge of its presence, is its power of activating pancreatic juice. If the trypsin solutions contain enterokinase, they also must possess a power of activating pancreatic juice. This indeed is stated by Vernon[1] to be the case, and we have therefore had to devote many experiments and much time to the decision of this point. The experiments on the activating power of trypsin are complicated by the fact that one is here adding a solution which is itself proteolytic,

[1] This *Journal*, XXVII. p. 288. 1901.

and a solution whose proteolytic power will vary according to the reaction of the fluid, the presence or absence of dissolved proteids in the fluid, and the reaction of the fluid. The first experiments on this point were made with Grübler's trypsin, comparing the activating powers of Grübler's trypsin with that of a solution of enterokinase. The results of the experiments are given below.

Effect of a solution of Grübler's trypsin on inactive juice.

				24 hrs.		24 hrs.
I.	*a.*	5 c.c. juice + 1 c.c. trypsin	All with gelatin tubes.	3 mm.	Fresh tubes added to *a* and *b*.	1 mm.
	b.	5 c.c. 1 % NaF + 1 c.c. trypsin		4 ,,		2·5 ,,
	c.	5 c.c. juice + 1 c.c. enterokinase		11 ,,		

II. *a.* 5 c.c. juice + 1 c.c. trypsin.
b. 5 c.c. 1 % NaF + 1 c.c. trypsin.

Both kept for 1 hr. at 40° C., then cooled, and gelatin tubes added.

Digestion after 20 hrs.—*a.* 2 mm.
b. 2 ,,

Enterokinase then added to both, and fresh gelatin tubes.

Digestion after 24 hrs.—*a.* 4·75 mm.
b. 2·5 ,,

Effect of Merck's trypsin on inactive glycerin extract of pancreas.

The extract, which did not digest gelatin, even on dilution, until the addition of enterokinase, and which will be described in a subsequent paper, was diluted 10 times with $\frac{N}{20}$ Na_2CO_3 and a part of it heated to 100° in a steam sterilizer.

a. 5 c.c. diluted glycerin extract + 1 c.c. trypsin.
b. ,, ,, ,, heated + ,, ,,
c. ,, ,, ,, extract + 0·2 trypsin + 0·8 $\frac{N}{20}$ Na_2CO_3.
d. ,, ,, ,, heated + ,, ,, + ,, ,,
e. ,, ,, ,, extract + 1 c.c. enterokinase.

Left for 24 hrs. at room temp. then gelatin tubes.

Digestion after 24 hrs.—*a.* 6·3 *b.* 6·1 *c.* 5·4
d. 5·6 *e.* 6·5

Then added 1 c.c. ent. to all.

Digestion after further 24 hrs. (warmer day)—

a. 8·2 *b.* 8·3. *c.* 6·1
d. 5·8 *e.* 4·9

Another experiment with weaker trypsin solution:—

a. 5 c.c. diluted glycerin extract + 0·2 trypsin + 0·8 c.c. $\frac{N}{20}$ Na_2CO_3.

b. ,, ,, ,, heated + ,, ,, + ,, ,,

c. ,, ,, ,, extract + 1 c.c. $\frac{N}{20}$ Na_2CO_3.

d. ,, ,, ,, ,, + 1 c.c. ent.

e. ,, ,, ,, ,, + 1 c.c. ent. + 0·2 c.c. trypsin.

Gelatin tubes in all.

Digestion after 17 hrs.—*a.* 3·0 *b.* 3·1 *c.* 0
d. 5·1 *e.* 6·0.

It will be seen that the pancreatic juice *plus* trypsin had no greater proteolytic power than a solution of sodium fluoride or sodium carbonate *plus* trypsin; in fact in some cases the proteolytic power of the pancreatic juice *plus* trypsin was less than that of a sodium carbonate solution containing the same strength of trypsin. There is no doubt that Grübler's trypsin has no activating power on pancreatic juice, and therefore contains no enterokinase. In other observations, one of which is recorded above, we have noticed that it can digest and destroy the precursor of trypsin, trypsinogen, without in any way converting it into trypsin. The hydrolytic splitting of trypsinogen, that is to say, does not necessarily involve its conversion into trypsin.

The experiments with an active glycerin extract of pancreas, though very similar to those just described, do present some slight indications of the presence of an activating substance, and we shall have to return to them later on. Experiments with Grübler's trypsin are sufficient to prove that trypsin is not a mixture of trypsinogen and enterokinase, but is a third substance which may exist in the absence of either of these two other bodies. It is a product of that interaction, and not merely the sum of their addition.

We have not been able to confirm Delezenne's observations on the affinity of fibrin for enterokinase. We have soaked flakes of fibrin in enterokinase solutions for a considerable time, but have not found that their intimate admixture with the fluid resulted in any diminution of the amount of enterokinase contained in the fluid. The fibrin certainly took up a small trace of enterokinase, and was therefore able to activate pancreatic juice, but this is what one would expect, and does not indicate any specific attraction of the fibrin for the enterokinase.

Fresh fibrin, wrung out as dry as possible, was moistened with an active solution of enterokinase, and allowed to stand for 48 hrs. At the end of this time the fluid was

squeezed out of the fibrin and its activating power compared with that of the original solution of enterokinase.

	Gelatin tubes after 24 hrs.
a. Juice + original enterokinase	10 mm.
b. „ + enterokinase squeezed out of fibrin	9 „

The fibrin was found to activate juice in which it was allowed to stand.

We must therefore conclude with Pawlow that enterokinase is a ferment, with a specific action on trypsinogen. When it has effected the conversion of trypsinogen into trypsin its office is finished, and it takes no further part in the action of trypsin on the proteid constituents of the fluid. It is, in fact, rapidly destroyed at the temperature of the body, especially in the presence of trypsin.

What is the nature of this action? If, as in the case of so many other ferments, it is merely a hydrolysis, we should expect to be able to imitate its effect by chemical means; and many physiologists have described various methods of activating pancreatic extracts, *i.e.* of converting trypsinogen into trypsin by simple chemical means, such as the addition of weak acids, oxygenation, etc.[1]

We have tried all the procedures we could think of for effecting this conversion but without success. We have already described our negative results as regards the action of Grübler's trypsin on trypsinogen. We have acidified the juice with weak or strong acid and then neutralised or made alkaline again after varying periods; we have left the juice in contact with fibrin, with yeast, with takadiastase, with platinum-black, with gastric juice in acid or alkaline media, with saliva, hydrogen peroxide, and sodium amalgam; most of these treatments the juice stood extremely well, *i.e.* it could be rendered active afterwards by the addition of enterokinase. In no case, however, did our treatment render the juice active without this addition. Moreover the juice showed no signs of spontaneous activation, even after standing for weeks, in the absence of infection by micro-organisms, and similar results have been lately obtained by Delezenne[2]. We are therefore obliged to look upon the action of enterokinase as absolutely specific. It is possible that a very slight "Umlagerung" in the molecule is sufficient to cause a conversion of trypsinogen into trypsin, and that the hydrolytic changes induced by pepsin or trypsin are too profound, leading at once to a total destruction of the active parts of the molecule. We have a parallel instance in the behaviour of

[1] Cp. Kühne, Heidenhain, &c.

[2] *C. R. Soc. de Biol.* 1903, p. 455.

tryptophane to organised ferments, and to chemical reagents respectively, lately described by Hopkins and Cole[1].

In view of the statement recently made by Vernon [2] and supported by many experiments, that trypsin itself is able to convert trypsinogen into trypsin, we have devoted special attention to this point. Although such a process would be not without analogy in other catalytic processes known to chemists, it would be quite a unique case among ferments that a product of a reaction should itself be the catalyser of the reaction. Such a reaction would necessarily progress with ever-accelerating velocity, and it should be possible by the study of the velocity of the change, apart from other considerations, to test the truth of Vernon's hypothesis. Although we have not made accurate determinations of the velocity of the change involved in the conversion of trypsinogen to trypsin, we have obtained no evidence of such increasing velocity of change as would be required by the hypothesis. Of course it must be remembered that our experiments differ in one important respect from Vernon's, in that we have always employed pure juice for our experiments, whereas Vernon's observations were made on the much more complex solutions obtained by macerating the fresh gland in various media. So far as the juice is concerned we have already given experiments to show the absence of any catalytic effect of Grübler's trypsin, and have mentioned there that this ferment will destroy the pro-ferment of the juice without apparently at any time converting it into ferment. Dr Vernon kindly placed at our disposal some of his active glycerin extracts of pancreas, which he had found to be rich both in trypsin and in the power of activating his pancreatic extracts.

The following series of observations shows the effect of this extract, in a 1 in 10 dilution, on pancreatic juice as compared with the action of enterokinase.

Effect of active glycerin extract of pancreas (Vernon) on inactive pancreatic juice.

(Trypsin = 1 in 10 solution of glycerin extract in 1 % NaF.)

			24 hrs.			24 hrs.
A.	1. 5 c.c. juice + 1 c.c. trypsin	All treated with gelatin tubes.	6 mm.			4·25 mm.
	2. 5 c.c. boiled juice + 1 c.c. trypsin		6 ,,		Fresh tubes.	3 ,,
	3. 5 c.c. diluted juice + 0·2 c.c. trypsin		2·5 ,,			3·75 ,,
	4. 5 c.c. boiled diluted juice + 0·2 c.c. trypsin		2·6 ,,			1·6 ,,

[1] This *Journal*, XXIX. p. 451. 1903.

[2] *loc. cit.*

			24 hrs.	24 hrs.		48 hrs.
B. 1.	5 c.c. juice + 0·2 c.c. trypsin	All treated	1	1	enterokinase added	4·2
2.	5 c.c. juice + 0·2 c.c. enterokinase	with	6	5	,,	4
3.	5 c.c. $\frac{N}{10}$ Na_2CO_3 + 0·2 trypsin	gelatin tubes.	2	2	,,	2·3

It will be seen that with fairly large quantities of this diluted extract, namely, 1 c.c. to 5 c.c. of juice, the proteolytic power of the mixture was the same whether the juice had been previously boiled or not; that is to say, in this strength the total proteolytic activity of the mixture was due to the added trypsin solution, for which the reaction of the pancreatic juice represented apparently the optimum. When smaller quantities of the trypsin were used, namely, ·2 c.c. of the diluted extract to 5 c.c. of the juice, the activating effect for the first 24 hours was almost *nil*, and the slight proteolytic power presented by this solution could be entirely ascribed to the active trypsin introduced. In fact in some cases the mixtures of juice *plus* trypsin were less active than a solution of $\frac{N}{10}$ sodium carbonate containing the same proportion of trypsin (·2 c.c.). When these solutions were left for two or more days the pancreatic juice *plus* trypsin was finally more active than a similar solution of trypsin in sodium carbonate. This might be ascribed to a slight activating effect of the trypsin on the pro-ferment, or to the retarding effect exercised on the auto-destruction of the ferment by the proteid present in the pancreatic juice. In view of the fact that other preparations of trypsin had no activating effect whatever, and that any activating power possessed by a given solution of trypsin has no relation whatever to the proteolytic or tryptic power of the solution inclines us to believe that the active glycerin extracts contained not only trypsin but also enterokinase, and that its slight activating effect is due entirely to the presence of this latter substance. This conclusion was borne out by another set of experiments. If trypsin itself activates trypsinogen, a specimen of pancreatic juice which has been activated by the addition of a small amount of enterokinase should possess an activating power on further portions of juice which should be much greater than that represented by the small trace of enterokinase it contains. That this is not the case is shown by the following experiment:—

5 c.c. of fresh juice was treated with ·4 c.c. of active enterokinase solution. This was kept at 35° C. for 30 minutes, then cooled to room temperature, and gelatin tubes inserted. The next day the gelatin tubes were completely digested. 5 c.c. of fresh juice were now treated with ·1 c.c. of this active juice. This amount would contain ·008 c.c. of the

original enterokinase. At the same time, another portion of fresh juice was treated with ·008 c.c. of fresh enterokinase. In 20 hrs. time the digestive powers of the fluids were almost identical; that treated with ·1 c.c. of active juice having digested 4·5 mm., while that treated with ·008 c.c. of fresh enterokinase digested 5·1 mm., and in a further 26 hrs. the digesting powers were still in the same, proportion, namely 6·5 mm., 7·2 mm. If the trypsin produced by the activation of the juice had had any activating power on the fresh juice, the digestive power of the first specimen should have been at least ten times as great as that of the specimen treated with the enterokinase alone.

We must conclude, therefore, that trypsin has no activating influence whatever on trypsinogen, and that the only substance capable of converting trypsinogen into trypsin is the enterokinase found in the small intestine. We hope to return in a future paper to the consideration of Vernon's results on pancreatic extracts, and the explanation of the reason of the apparent discrepancy between the behaviour of trypsinogen in a gland extract, and that of trypsinogen as contained in the fresh pancreatic juice.

Source of enterokinase.

We have already mentioned the view put forward by the French school—Delezenne, Metchnikoff[1] and others—that enterokinase is merely one of the class of cytases, and that it is formed in the leucocytes in the body generally, both in the blood as well as in those of the mucous membrane of the alimentary canal. Delezenne[2] states that active enterokinase can be obtained from blood leucocytes, from lymphatic glands, and especially from Peyer's patches, and regards the source in the intestine as the leucocytes of the mucous membrane, especially of the Peyer's patches. He ascribes the solution of blood fibrin by fresh juice to the presence in the fibrin of a trace of enterokinase derived from disintegration of blood leucocytes. We have repeated the experiments of Delezenne very many times, but are quite at a loss to account for his conclusions. Although fibrin obtained from blood does dissolve in fresh pancreatic juice, it does not lend to this juice any increased proteolytic power. When the solution is complete the mixture is as unable to digest boiled white of egg or gelatin as it was before the addition of the fibrin. With fibrin derived from the slaughter-house it may be possible sometimes to observe a slight activating influence, but the activity of enterokinase is so great (·0001 c.c. of an active extract will activate 5 c.c. of pancreatic juice

[1] *L'Immunité dans les maladies infectieuses*, Paris, 1901, p. 65.

[2] *C. R. Soc. de Biologie*, 1902, p. 282.

in 2 to 3 days) that the slightest contamination with intestinal contents, such as may occur from the hands of the slaughter-man, will suffice to impart an activating power to almost any tissue or fluid. Extracts made in various ways from lymphatic glands, either of the abdomen or of the neck, are absolutely without influence on the pancreatic juice. Moreover, the distribution of the enterokinase in the intestine does not lend any support to the view that it is derived from the leucocytes of the intestine. In the following experiments we give the results of attempts made to activate pancreatic juice by various extracts of the intestine and lymphatic glands.

Comparison of activating powers of extracts of various tissues on pancreatic juice.

The extracts were made by rubbing up weighed portions of the tissues in ten times their weight of toluol water. The mixtures were then left for 20 hrs. at room temperature, and the next day were filtered and the filtrate added to an equal bulk of fresh juice, and gelatin tubes added. All the vessels and instruments used were previously carefully sterilised.

		20 hrs.	40 hrs.
a.	Juice + first scraping duodenum (mucus and epithelium)	7 mm.	all
b.	,, + second scraping duodenum (epithelium and leucocytes)	6 ,,	,,
c.	,, + first scraping lower six inches ileum	3 ,,	,,
d.	,, + Peyer's patches from which the epithelium had been scraped	0	0·8
e.	,, + abdominal lymphatic glands	0	0

In another experiment the extract was made with 2 % NaF, the rest of the experiment being carried out as before.

The following extracts had *no* activating influence even after 40 hrs., viz. cervical lymphatic glands, abdominal lymphatic glands, spleen, liver.

The following gave positive results:

	Digestion after 17 hrs.	Digestion after 40 hrs.
First scraping duodenum	3 mm.	all (*i.e.* more than 12)
Second scraping duodenum	0 ,,	1·2 mm.
First scraping ileum	0·5 ,,	11 ,,
Peyer's patches (with epithelium)	3 ,,	all
Diluted and filtered succus entericus from duodenum	10 ,,	all
Ditto from ileum	10 ,,	all

It will be seen that in the intestine the greatest activating power is possessed by the succus entericus. Next in order come the solutions made by extracting the first scraping of the mucous membrane, *i.e.* the scraping which contains chiefly epithelial cells, together with the mucus adhering to the walls of the intestine. Here again there is a

difference between different parts of the gut, the extract from the scraping from the duodenum being usually more potent than a similar extract made from the lower end of the ileum. If the intestine after the first scraping be washed under the tap and then scraped again, an extract of this second scraping is almost without effect on pancreatic juice. This preponderating presence in the succus entericus is in distinct contrast to the distribution of secretin, and leads us to believe not only that the leucocytes are absolutely free from enterokinase, but also that in all probability the enterokinase does not exist preformed in the epithelial cells, as does prosecretin, but that it is first formed as the result of a secretory activity of these cells. We thus arrive at the conclusion that enterokinase is perfectly specific in its distribution, and in its action. Its sole action is the transformation of trypsinogen into trypsin, and it is formed only in that portion of the body where this effect can be of use to the organism. Moreover, it is the only substance which has any activating effect on pancreatic juice. The results obtained by the French school are probably due, in part at any rate, to the fact that pancreatic juice contains two substances, namely, a weak proteolytic ferment similar to erepsin, which is preformed, and a body, trypsinogen, which is without effect on proteids, but which can be converted by enterokinase into the most active proteolytic ferment known.

Preparation and properties of enterokinase.

We have found that the best method of preparing enterokinase is to lightly scrape with the handle of a scalpel the upper part of the mucous membrane of the small intestine, to digest the scrapings with chloroform water for a couple of days, and then to filter the mixture first through paper, and then through a Berkefeldt filter. The resulting solution is perfectly clear, contains a certain amount of coagulable proteid, and will retain its activity at the room temperature for many months. Although so stable at room temperature it is very rapidly destroyed at 35° to 40° C. Thus, in one experiment 5 c.c. of pancreatic juice was treated with 1 c.c. of enterokinase solution which had been kept at 40° C. for 24 hours. Another specimen of juice was treated with the ordinary solution of enterokinase not heated, and gelatine tubes were placed in each. At the end of 20 hours only 1 mm. of gelatin was digested in the juice with the warmed enterokinase, while in the other specimen of juice 10·25 mm. were digested. In this respect the

enterokinase resembles trypsin itself, which, as shown by Vernon, is extremely rapidly destroyed at a temperature of 35° to 40° C.

It has been stated by Stassano et Billon[1] that the enterokinase in extracts of intestinal mucous membrane is bound up, if not identical, with the nucleo-proteids. This statement we have not been able to confirm. Thus, in one experiment the watery extract of mucous membrane was precipitated with acetic acid, and the nucleo-proteid was extracted with alkaline solution. It only dissolved to a slight extent. The insoluble part was suspended in a weak solution of sodium fluoride. Three specimens of pancreatic juice were then treated with (1) 1 c.c. of the acid solution free from nucleo-proteid, (2) a suspension of insoluble nucleo-proteid, (3) with the alkaline extract of nucleo-proteid; and gelatine tubes were added to each. The digestion at the end of 20 hours was 9 mm. in the case of (1), 7·5 mm. in the case of (2), and only 1·5 in the case of (3), *i.e.* the acid solution free from the greater part of its nucleo-proteid had preserved the greater part of the enterokinase.

The enterokinase solution is rendered inactive by heating for 5 minutes at 65° C., and is robbed of the greater part of its activity by similarly heating to 60° C. As indicated above, it has an extraordinary power of influencing the pancreatic juice. In one experiment 0·0001 c.c. of an extract made with chloroform water, and containing 1 % of total solids, chiefly proteids, was able to activate 5 c.c. of pancreatic juice in 3 days. This great activity of enterokinase renders it important in experiments on the subject to take special precautions as regards cleanliness of vessels and instruments used, since the slightest contamination with intestinal contents or mucous membrane may introduce sufficient enterokinase to cause activation of an inactive pancreatic juice.

The determination of the tryptic power of pancreatic juice.

A number of researches have been made by the Pawlow school on the proteolytic activity of pancreatic juice secreted under various conditions. In these researches advantage was taken of the "law of squares" enunciated by Schütz and Borissow, according to which the amount of substance—egg albumen or gelatin—dissolved in a given time varies according to the square root of the amount of ferment present. Delezenne has shown that in all these cases the slight

[1] *C. R. Soc. de Biol.* 1902, p. 623.

amount of trypsin present was due to the fact that the juice was contaminated with varying proportions of enterokinase as it flowed over the mucous membrane in the fistula. The fact that these two factors, enterokinase and trypsinogen, are both involved in the tryptic power of the secreted pancreatic juice, renders it almost impossible to determine accurately the amount of trypsinogen in a given specimen of juice. If we are dealing with pure solutions of trypsin, the law of squares is sufficiently accurate for practical purposes, especially if the observation be confined to a short period of time, and be carried out on gelatin tubes at the temperature of the room, so as to avoid a too-rapid auto-destruction of the ferment. The following are the results which we obtained with solutions of Grübler's trypsin.

Relation of ferment action to amount of ferment.

(Trypsin = strong solution of Grübler's trypsin in 1 % NaF.)

		24 hrs.	48 hrs.
a.	20 c.c. 1 % NaF + 0·1 c.c. trypsin	·9 mm.	1·7 mm.
b.	,, ,, + 0·4 c.c. ,,	1·8 ,,	3·3 ,,
c.	18·4 c.c. ,, + 1·6 c.c. ,,	3·0 ,,	5·6 ,,
d.	13·6 c.c. ,, + 6·4 c.c. ,,	4·0 ,,	7·9 ,,

The approximation to the theoretical figures required by the "law" is only a rough one, and it is evident that the complexity of the processes involved, especially the auto-destruction accompanying the activity of the ferment, must deter us from making any general conclusions from the figures obtained.

When, however, we are dealing with fresh pancreatic juice, to which we add enterokinase, the process becomes much too complex for easy determination of the ferment activity. We have at least 4 processes going on, the conversion of trypsinogen into trypsin, an effect of the trypsin so produced on the proteid molecule, an auto-destruction of enterokinase, and an auto-destruction of the trypsin. Although it is of great importance that we should have some means of estimating the total amount of trypsinogen present in any given specimen of juice, especially if we are to repeat Walther's experiments on the adaptation of the gland to the diet, we have been unable, so far, to hit upon any condition of experiments which will give us any numerical expression for the trypsinogen present. A strong solution of trypsinogen when treated with a fair amount, say $\frac{1}{5}$, of an enterokinase solution will digest more rapidly than a weak solution; but beyond this we cannot go. We thought that possibly we might get some proportionality between

the amount of ferment and tryptic power if we added a very large excess of enterokinase. Thus, in one experiment 5 c.c. of enterokinase were treated with ·1 c.c., ·4 c.c., and 1·6 c.c. of juice. The resulting digestion at the end of 20 hours was represented by 6, 8·2, and 10 mm. of gelatin. It is possible that if the experiment had been of shorter duration we might have obtained results more in accordance with the law of squares, since a similar experiment made with egg-albumen at 40° C. gave as results of digestion ·5, 1, and 1·5 mm., *i.e.* the proportions required by the law of squares. It is certain, however, that any experiments on the proteolytic power of a juice which are conducted in the absence of a very large excess of enterokinase must be absolutely worthless so far as regards any indications they may give of the amonnt of trypsinogen present.

Conclusions.

1. Under no circumstances does the pancreatic juice, as secreted, contain trypsin.

2. Fresh pancreatic juice contains trypsinogen, and a weak proteolytic ferment resembling somewhat erepsin. This latter will digest fresh fibrin, or caseinogen, but has no influence on coagulated proteid or on gelatin.

3. Trypsinogen is a stable body, only slowly altered by standing in alkaline or acid solutions. It is converted into trypsin by the action of enterokinase. No other substance can effect this conversion.

4. Trypsin is not an expression for two bodies, enterokinase and trypsinogen acting together, but is a third substance produced as a result of the interaction of those two bodies, *i.e.* enterokinase acts on trypsinogen like a ferment (Pawlow) and converts it into trypsin.

5. Trypsin is extremely unstable, and is rapidly destroyed, especially in alkaline media, and at the body temperature (cp. Vernon). This auto-destruction is greatly retarded by the presence of dissolved proteids or peptones.

6. Enterokinase is a product of secretion of the small intestine, and mainly of its upper end, but of no other parts of the body. It is not found in blood fibrin, lymphatic glands, or leucocytes.

7. The smallest trace of enterokinase will convert a large quantity of trypsinogen into trypsin if sufficient time be allowed. It is stable in watery solutions at 15° C., but is rapidly destroyed at 40° C.